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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 4 : G01N 33/53, 33/531, 33/532 G01N 33/543, 33/571, 33/574 G01N 33/577, 33/569

A1

(11) International Publication Number: WO 87/ 06005

(43) International Publication Date: 8 October 1987 (08.10.87)

(21) International Application Number: PCT/US87/00577

(22) International Filing Date: 20 March 1987 (20.03.87)

(31) Priority Application Number: 843,437

(32) Priority Date: 24 March 1986 (24.03.86)

(33) Priority Country: US

(60) Parent Application or Grant
(63) Related by Continuation
US 843,437 (CIP)
Filed on 24 March 1986 (24.03.86)

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(81) Designated States: AT (European patent), AU, BE (European patent), CH (European patent), DE (European patent), DK, FR (European patent), GB (European patent), IT (European patent), JP, KR, LU (European patent), NL (European patent), SE (European patent), US.

Published
With international search report.

(54) Title: SYNTHETIC HTLV-III PEPTIDES, COMPOSITIONS AND USES THEREOF

(57) Abstract

Synthetic peptides useful for detection of antibodies to HTLV-III virus, and diagnostic and therapeutic compositions and methods of use.

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SYNTHETIC HTLV-III PEPTIDES, COMPOSITIONS AND USES THEREOF

This is a continuation-in-part of our patent application Serial Number 843,437 filed on March 24, 1986.

FIELD OF THE INVENTION

The subject invention relates to synthetic peptides and, more particularly, to synthetic peptides which mimic a portion of a protein or proteins produced by the HTLV-III or HTLV-III-like viruses that are etiologically associated with the disease syndromes known as AIDS and ARC.

BACKGROUND OF THE INVENTION

Acquired Immune Deficiency Syndrome (AIDS) was first recognized in the United States in 1981, and the number of United States cases has been doubling approximately every ten months since then. Since 1981 there have been about 16,000 reported AIDS cases in the United States, of which approximately half have already died. The expected outcome of the disease is invariably death, since there is currently no known treatment which can effectively delay or prevent the ravages of the disease. Although the disease first manifested itself in homosexual or bisexual males and intravenous drug abusers, it has now spread to others by shared use of contaminated needles or by intimate sexual contact with or receipt of blood products from a carrier of the virus.

The etiological agent associated with AIDS has been identified as a group of related retroviruses variously known as Human T-cell Lymphotropic Viruses-type III (HTLV-III), Human Immunodeficiency Virus (HIV),

Lymphoadenopathy Viruses (LAV) or AIDS-Related Viruses (ARV). These viruses will be collectively referred to herein for convenience as "HTLV-III viruses".

Because AIDS can be transmitted by blood products, there has, from the initial recognition of the disease, been a strong impetus to develop diagnostic tests to screen blood for antibodies or antigens specific for the infecting virus. Efforts in this area have borne fruit, and by the end of 1985 five companies had been approved to market tests to detect antibodies to HTLV-III virus. These tests all rely for detection of the antibodies on the use of viral proteins obtained from cultured HTLV-III infected T-lymphocytes. The virus obtained from the cultured cells is disrupted (e.g., with detergent) and a fluid (called "viral lysate") is obtained. This lysate (containing a variety of fragments of viral protein) is then typically used as the solid phase component of an immunoassay.

The current commercial immunoassays are of the conventional sandwich ELISA format, in which the solid phase component (having the viral lysate deposited thereon) is contacted with blood or serum suspected of containing HTLV-III antibodies. If the antibodies are present, they are expected to bind to the viral lysate and, after unbound material is washed away, are contacted with enzyme-labeled anti-human immunoglobulin. The labeled antibodies will bind to any human antibodies attached to the solid phase; the specificity of the test for HTLV-III antibodies is therefore conferred by the viral lysate.

While the existing tests appear to have significantly diminished the transmission of HTLV-III virus via blood

products, these tests based on viral lysate have some significant disadvantages.

First, because the viral lysate is produced from cells infected with live HTLV-III virus, there is a possibility that those making the test might be infected during manufacture. There is also at least the theoretical possibility that the live virus might survive the disruption procedure and find its way into the diagnostic test and thus infect the user.

A second disadvantage involves the difficulty of obtaining the lysate and the variable nature of the resulting lysate depending on variation in processing procedure or in characteristics of the infected cells used to obtain the viral lysate.

A third, and far more serious, practical disadvantage is the substantial number of false positive and, to a lesser extent, false negative results observed using the current tests. It is by now well recognized that the current viral lysate ELISA tests yield a substantial number of false positive results. As observed in the Hastings Center Report, Special Supplement/August 1985, entitled "AIDS: The Emerging Ethical Dilemmas":

> "However, the ELISA test is not as specific as it is sensitive; that is, in a population of healthy blood donors, as many as one out of every hundred test results will be positive; and from these, as many as 90 out of 100 will be falsely positive. On a national scale, out of the 8 million blood donations each year, 40,000 will be falsely positive." (Page 9)

The false positives are thought to be due in part to the presence of non-viral proteins in the viral lysate preparations used in the solid phase component of the current assays.

The report further indicates that a Western blot confirmatory test (which is more expensive and more technically difficult than the ELISA test) must be performed to exclude the false positive results. Since Western blot assays are themselves subject to error and subjective interpretation, a simple, quick and objective confirmation test for HTLV-III antibody is still desirable.

False negatives are also a concern, although such false negatives may result partially from the nature of the assay, from the immunosuppressive nature of the disease, or from the latency period between exposure to HTLV-III virus and development of antibody.

A further disadvantage of the currently-available tests is that they detect only antibody to the HTLV-III virus and not the virus or viral antigen itself. A positive result therefore indicates only that the tested subject was at one time exposed to the HTLV-III virus and developed antibodies thereto; it is inconclusive about whether the subject is currently infected, or is infectious, or has AIDS. It should be noted that in the revised Center for Disese Control definition of AIDS (June 1985) patients are excluded as AIDS cases if they are negative for serum antibody to HTLV-III and do not have a low number of T-helper lymphocytes or a low ratio of T-helper to T-suppressor lymphocytes.

Despite their inconclusive diagnostic value, the current tests are being used not only to detect

virus-contaminated blood, but also to detect infected persons (whom it is assumed can possibly progress to frank AIDS and transmit the disease to others). Because a substantial number of people who are positive in the commercially-available antibody tests neither exhibit clinical symptoms nor (apparently) are capable of infecting others, there is a substantial risk with current tests of falsely identifying a person as an AIDS carrier with the consequent social and psychological results.

Many of the disadvantages of the current lysate-based test could be obviated by substituting for the viral lysate a material not derived from virus-infected cells. Such a material could be, for example, a virus-specific synthetic peptide or a virus-specific peptide derived from a recombinant organism (typically E. Coli).

Work on the latter approach has been reported by Robert C. Gallo and coworkers in a variety of recent articles, including Biotechnology, Vol. 3, Pages 905-909 (October 1985), Science, Vol. 228, Pages 93-96 (April 5, 1985), and Nature, Vol. 315, Pages 151-154 (May 9, 1985). These workers have identified an 82 amino acid peptide encoded by a gene segment in the ENV region of the HTLV-III virus produced through recombinant E. Coli techniques. This peptide is recognized by antibodies to HTLV-III virus. More recently, a Genentech group has reported work on a 102 amino acid peptide containing within it the 82 amino acid peptide of the Gallo group. Biotechnology, Vol.4, Pages 128-133 (February 1986)

While the synthetic peptide technique offers more apparent advantages (e.g., specificity, purity, ease of preparation, and the like) there appears to have been little work in this area. In fact, the only publication

of which the present inventors are aware that relates to such work is a presentation by Dr. Dino Dina and coworkers at the Fifth Annual Congress for Recombinant DNA Research, February 3-6, 1985. While the Abstract of the presentation indicates that various synthetic peptides "are being used (1) to assess immune responses in AIDS patients and (2) to raise antisera in animals", the specific peptides used were not disclosed. Moreover, in the presentation itself, no specific peptides were identified and no indication was given that any one peptide or combination of peptides could recognize all or most of the known positive sera. Since that presentation in February 1985 and up to the date of filing the parent patent application on which this application is based, we are aware of no further publications by that research group or any other on synthetic HTLV-III peptides. Subsequently, in August of 1986 a publication by Wang et al., Proc. Natl. Acad. Sci., U.S.A., (1986) 83:6159-6163 was published.

Synthetic peptides which would successfully mimic a portion of the HTLV-III protein and hence would be useful both for detection of antibody to HTLV-III and for production of antibody which would recognize HTLV-III virus or viral antigen would be a significant advance in the art. The present invention provides such peptides, compositions containing such peptides, and methods for using these peptides and compositions for therapy and diagnosis. The invention also provides anti-peptide antibodies, compositions containing these antibodies, and methods for using the antibodies and compositions.

SUMMARY OF THE INVENTION

The present invention provides synthetic peptides each

of which have a sequence of at least seven amino acid residues corresponding to residue sequences within the gp 41 envelope (ENV) protein of the HTLV-III virus. Such peptides have been found to mimic the immunological properties of the native virus itself and are selected from the group consisting of:

CSGKLIC (I)
IWGCSGKLICTTAVP (II)
IWGCSGKLICTTAVPWNAS (III)
AVERYLKDQQLLGIWGCSGKLI (IV)
AVERYLKDQQLLGIWGCSGKLICTTAVPWNAS (V)
LKDQQLLGIWGCSGKLI (VI)
LLGIWGCSGKLIC (VII)
QQLLGIWGCSGKLICTTAVPWNAS (VIII)
IWGCSGKLICTTAVPWN (IX)
CSGKLICTTAVPWNAS (X)
SGKLICTTAVPWNAS (XI)
AVERYLKDQQLLGIWGCSGKLIC (XII)
GCSGKLICTTAVPWN (XIII)
LKDQQLLGIWGCSGK (XIV)
RILAVERYLKDQQLLGIWGCS (XV)

These peptides are recognized by a majority of HTLV-III antibody-positive sera from patients with AIDS/ARC, as well as antibody-positive sera of unknown diagnosis. In addition, the subject peptides yield hardly any false positives. The preferred peptides which are recognized by substantially all HTLV-III anti-positive sera are those of formulas (II) through (VI), (VIII), (X) and (XII) through (XV).

These results are highly surprising in view of the fact that other peptides corresponding to portions of the HTLV-III envelope proteins are ineffective to selectively

recognize antibodies to HTLV-III virus.

The present invention further comprises the discovery that recognition of antibodies to HTLV-III virus is significantly enhanced if the above peptides are used in combination, conforming to a specific selection perscription. Specifically, it has been discovered that enhanced recognition is achieved by selecting two or more of the above described peptides wherein a first of said peptides contains the gp 41 protein sequence:

CSGKLIC (I)

Preferably this first peptide contains at least these seven amino acid residues and still more preferably at least a twelve amino acid residue sequence from the gp 41 protein and including the sequence of formula (I).

The combination of peptides should also include at least a second peptide wherein said peptide includes at least the amino acid residue sequence of

LLG(X)W (XVI)

wherein X is selected from the group consisting of I, L, M or F. It should be noted that X corresponds to the 602 position of the gp 41 protein and when X is I, the formula (XVI) sequence corresponds to the 599 to 603 position of the gp 41 protein. On the other hand, it is believed that based on a study of the sequence comparison of HIV isolates in the critical region of the gp 41 protein, substitution for I in this position, 602, with L, M or F are antigenetically equivalent. This second peptide, in addition to including the sequence of formula (XVI), should also include a total of at least fifteen

amino acid residues in a sequence found in the gp 41 protein. Stated in other words, the second peptide comprises the sequence

ZLLGXWZ';

wherein X is selected from the group consisting of I, L, M or F;

wherein Z is selected from the amino acid residue sequence of the HTLV-III virus gp 41 protein immediately adjacent to the amino side of the L-leucine residue in the 599th position of the gp 41 protein;

wherein Z' is selected from the amino acid residue sequence of the HTLV-III virus gp 41 protein immediately adjacent to the carboxy side of the L-tryptophan residue in the 603rd position of the gp 41 protein; and

wherein one of Z or Z' may be zero residues long and wherein Z and Z' together comprise at least ten residues.

The combination of peptides (IV) or (XII) with peptides (III), (XIII), or (X) are especially preferred. The combination of (III) and (IV) or (XIII) and (IV) being the combinations of choice.

In view of these results, it is clear that a significant antigenic determinant of the HTLV-III virus which reacts with HTLV-III antibodies is contained within the seven amino acid residue peptides that include the sequence of formula (1) described above. Moreover, even though each of the subject peptides reacts with most HTLV-III positive sera, individual patient sera have been observed to react specifically with one of the subject

peptides but not another. This observation indicates that additional antigenic determinants exist in longer peptides containing the sequence of formula (I), such as peptides (II) through (XI). It is well within the skill of an ordinary worker in the peptide synthesis art to prepare fragments of the subject peptides to determine antigenic and immunogenic fragments within them. Accordingly, such antigenic and immunogenic fragments are considered part of the present invention. Moreover, one of skill would also recognize that longer peptides corresponding to a portion of the HTLV-III envelope peptide and conforming to the teachings herein would function in the practice of the subject invention. Accordingly, such longer peptides are also considered part of the present invention.

The peptides of the invention contain at least one cysteine residue, and in certain instances two of such residues. Accordingly, the subject peptides may exist in various oxidative forms. In addition to the monomeric form in which the sulfhydryl group of the cysteine residue(s) is reduced, there may also exist dimeric or polymeric forms in which sulfhydryl groups on two or more peptide molecules become oxidized and form disulfide bonds. While subject peptides that possess only one cysteine residue can form only linear dimers, those that possess two cysteine residues may form cyclic monomers or linear or cyclic dimers and linear polymers of various lengths. These various oxidative forms are considered part of the subject invention are are included in the terms "subject peptides".

The present invention further provides a method of detecting or determining HTLV-III antibodies and a diagnostic kit for detection or determination of HTLV-III antibodies using one or more synthetic peptides of the

invention. The subject method for detecting or determining antibodies to HTLV-III in a fluid suspected of containing said antibodies e.g. blood, serum, plasma, saliva or urine, comprises the steps of:

a) providing at least one subject peptide or an antigenic fragment thereof attached to a solid surface;

b) contacting said peptide-coated solid surface with the fluid to be tested for a sufficient time to allow an immunologic reaction to occur; and

c) detecting or determining the presence or amount of antibodies bound to said peptide or antigenic fragment.

It may be useful to separate the solid surface from said fluid and to wash unbound material from the solid surface material after step b, depending on the detection method employed. While the specific detection technique is not critical, enzyme labelled anti-human immunoglobulin has been found to perform well. The subject diagnostic kit for practicing this method comprises a solid surface having at least one subject peptide or an antigenic fragment thereof bound thereto and labelled (preferably enzyme labelled) anti-human immunoglobulin. Other conventional materials for labelling antibody may also be used, as for example, biotin or a radioisotope.

In addition, the present invention provides a method of preparing anti-peptide antibodies useful for detection of HTLV-III virus or viral antigen which comprises immunizing a host animal with a subject peptide or an immunogenic fragment thereof in polymerized form or

attached to a suitable immunogenic carrier. The method for preparing polyclonal antibodies to HTLV-III comprises immunizing a host animal with at least one subject peptide or an immunogenic fragment thereof in polymerized form or attached to a suitable immunogenic carrier and bleeding the host. The method for preparing monoclonal antibodies to HTLV-III comprises immunizing a host animal with at least one subject peptide or an immunogenic fragment thereof in polymerized form or attached to a suitable immunogenic carrier, isolating the splenocytes from said immunized host, fusing said splenocytes with a suitable myeloma cell line, selecting the fused cells by reactivity to the immunizing peptide or fragment, to HTLV-III, or to HTLV-III-infected cells, and either culturing the selected hybridoma in vitro or injecting it into a suitable host. The resulting desired monoclonal antibody may be recovered from the supernatant over the cultured hybridoma or from the serum or ascites of the innoculated host.

The anti-peptide antibodies themselves, the method of detecting HTLV-III virus using the antibodies, and diagnostic kits containing said antibody (useful for detecting HTLV-III virus) are also included within the subject invention.

A sandwich method of detecting or determining HTLV-III virus or viral specific antigens in a fluid suspected of containing said virus or antigen comprises the steps of:

a) providing antibody to at least one subject peptide or an immunogenic fragment thereof, said antibody being attached to a solid surface;

b) contacting said antibody-coated solid surface with the fluid to be tested for a sufficient time

to allow an immunologic reaction to occur; and

c) detecting or determining the presence or amount of HTLV-III virus or viral specific antigens attached to said antibodies on said solid surface.

It may be useful to separate the solid surface from the fluid to be tested and to wash away unbound material from the solid surface after step b, depending on the detection method employed.

A competition method of detecting or determining HTLV-III virus or viral specific antigen in a fluid suspected of containing said virus or antigen comprises the steps of :

a) providing an antibody to at least one subject peptide or an immunogenic fragment thereof, said antibody being attached to a solid surface;

b) mixing an aliquot of the fluid to be tested with a known amount of labelled subject peptide to produce a mixed sample;

c) contacting said antibody - coated solid surface with said mixed sample for a sufficient time to allow an immunologic reaction to occur;

d) separating the solid surface from the mixed sample;

e) detecting or determining the presence or amount of labelled peptide either bound to the solid surface or remaining in the mixed sample; and

f) determining from the result in step e) the presence or amount of said virus or antigen in said fluid.

The diagnostic kit for detecting or determining HTLV-III virus by sandwich or competition method comprises:

a) a solid surface having bound thereto antibody to at least one subject peptide or an immunogenic fragment thereof; and

b) a known amount of labelled antibody to HTLV-III, a viral specific antigen, a subject peptide, or an immunogenic fragment thereof (for a sandwich assay); or a known amount of a labelled subject peptide (for a competition assay).

Still further, the invention includes prophylactic and therapeutic applications of the subject peptides. In one of these applications, the invention includes a method of immunizing an animal against HTLV-III virus which comprises parenterally administering to said animal an immunogenically-effective amount of at least one subject peptide or an immunogenic fragment thereof in polymerized form or attached to an immunogenically-effective carrier. In a second aspect of the therapeutic application of the subject invention, the invention includes a method of treating an animal having latent or actual HTLV-III viral infection which comprises parenterally administering to said animal an amount of at least one peptide of the invention or an immunogenic fragment thereof in polymerized form or attached to an immunogenically-effective carrier, said amount being effective to treat the HTLV-III viral infection. In still another therapeutic application, antibodies raised against

the subject peptides can be linked to toxic or medicinal substance and directed against infected cells. A final therapeutic aspect of the present invention is a composition of matter comprising an immunogenic amount of at least one peptide of the invention or an immunogenic fragment thereof in polymerized form or attached to an immunogenically-effective carrier, suitable for use in either of the above methods.

A subject peptide or an antigenic fragment thereof or a recombinant HTLV-III protein may also be used to improve the specificity of the subject peptide test or one using recombinant HTLV-III protein and thus reduce reliance on the more difficult Western blot confirmatory test. The accuracy of a positive result in the subject peptide test or a recombinant protein test may be confirmed by repeating the test in the presence of an effective blocking amount of a subject peptide or an antigenic fragment thereof or a recombinant HTLV-III protein. If binding of the supposed HTLV-III antibodies to the plate is blocked by the added peptide, fragment, or protein, the original positive result is confirmed; if binding is not blocked then the original result was a false (non-specific) positive.

The present invention therefore also includes a method of determining the accuracy of a positive result of a test for HTLV-III antibodies on a fluid sample suspected of containing said antibodies, said test being a sandwich assay in which a recombinant HTLV-III protein or synthetic HTLV-III peptide is attached to the solid surface, which method comprises the steps of:

a) mixing an aliquot of said sample with an effective blocking amount of a subject peptide or

an antigenic fragment thereof or recombinant HTLV-III protein to produce a mixed sample;

b) contacting said mixed sample with the solid phase of said test for a sufficient time to allow an immunologic reaction to occur;

c) determining whether binding of said antibodies in said mixed sample with said solid phase is inhibited compared to said binding in the absence of said peptide, fragment, or protein; and

d) determining, based on the presence or absence of said inhibition, the accuracy of said positive result.

The phrase "effective blocking amount" of the subject peptide, fragment or protein means an amount which will substantially completely react with any HTLV-III antibodies directed against the antigenic determinant(s) contained in said peptide, fragment, or protein that are present in the tested sample and thus substantially block reaction of these antibodies with the peptide or protein on the solid surface of the assay. The sandwich assay is preferably an ELISA assay.

The subject peptides may be prepared by any conventional technique (including ^{r}DNA technology and liquid phase synthetic techniques), although solid-phase Merrifield-type synthesis is a convenient way of preparing and isolating the peptide. A further description of this technique and of other art-known techniques may be found in the literature, i.e., M. Bodanszky, et al., Peptide Synthesis, John Wiley & Sons, Second Edition, 1976, as well as in other reference works known to those skilled in

the art. Synthetic techniques (as opposed to ^{r}DNA techniques) are preferred for reasons of purity, antigenic specificity, freedom from undesired side products, ease of production, and the like. Appropriate protective groups usable in such syntheses and their abbreviations will be found in the above text, as well as in J.F.W. McOmie, Protective Groups in Organic Chemistry, Plenum Press, New York, 1973. Both of these books are incorporated herein by reference.

Typical carriers to which the subject peptides may be attached for generation of anti-peptide antibodies or for preparation of therapeutic forms of the subject peptides include, e.g., bovine serum albumin; tetanus toxoid; keyhole limpit hemacyanin; porcine, bovine or equine immunoglobulin, and cholera or E. coli heat-labile toxin B-subunit.

To prepare the therapeutic compositions of the present invention, a subject peptide or fragment in polymerized form or attached to a suitable immunogenic carrier is combined as the active ingredient in intimate admixture with a parenterally-acceptable pharmaceutical carrier. This carrier will usually comprise sterile water, although other ingredients to aid solubility or for preservation purposes (e.g., thimerosal or methyl parabens) may be included. Injectable suspensions may also be prepared, in which case appropriate liquid carriers, suspending agents, and the like may be employed. These compositions may also contain an immunostimulator such as Thymopentin or Interleukin II or other adjuvants such as aluminum hydroxide or B.C.G.

The structures of the subject peptides are given in the conventional single-letter codes for amino acids and

are to be read, from left to right, as directionally corresponding to the amino to carboxy direction of the sequence. For the convenience of the reader, the single-letter codes for the amino acids contained in the subject peptides are:

I=L-isolucine; W=L-tryptophan; G=glycine; C=L-cysteine; S=L-serine; K=L-lysine; L=L-leucine; T=L-threonine; A=L-alanine; V=L-valine; P=L-proline; D=L-aspartic acid; N=L-asparagine; E=L-glutamic acid; R=L-arginine; Y=L-tyrosine; Q=L-glutamine; M = L-methionine; and F = L-phenylalanine.

The present invention is illustrated by the following examples.

EXAMPLE I

A. Synthesis of BOC-Proline Resin:

Chloromethylated styrene-divinylbenzene polymer containing 1.3 meq chloride/gram of resin was esterified with Boc-proline in anhydrous N,N-Dimethylformamide (DMF) using potassium iodide (KI) as the catalytic agent. The reaction was done at 55°C for 24 hours (1).. The substitution of Boc-Proline was 0.92 mMole/gram as determined using a picric acid assay on a portion of deblocked resin.

B. Synthesis and Characterization of Peptide (II).

Synthesis of the peptide of formula (II) was accomplished using classical Merrifield technique (2). The peptide sequence 'IWGCSGKLICTTAVP' was synthesized on a Vega 250C automated peptide synethsizer using a double

couple program. 1.8326g of Boc-proline resin was sequentially double coupled with the following Boc-L-amino acids (3,8) in twelve meq excess:

Amino Acid	Solvent
Boc-Val	CH_2Cl_2
Boc-Ala	CH_2Cl_2
Boc-(O-Bzl)-Thr	CH_2Cl_2
Boc-(O-Bzl)-Thr	CH_2Cl_2
Boc-(MeOBzl)-Cys	CH_2Cl_2
Boc-Ile	CH_2Cl_2
Boc-Leu	10% DMF/CH_2Cl_2
Boc-(Cl-Z)-Lys	CH_2Cl_2
Boc-Gly	CH_2Cl_2
Boc-(O-Bzl)-Ser	CH_2Cl_2
Boc-(MeOBzl)-Cys	CH_2Cl_2
Boc-Gly	CH_2Cl_2
Boc-Trp	10% DMF/CH_2Cl_2
Boc-Ile	CH_2Cl_2

The peptide was cleaved from the resin with 10% anisole in hydrofluoric acid and extracted with 20% aqueous acetic acid. This solution was filtered to remove solid resin and run through a Fractogel TSK HW-40F desalting column using an eluent of 20% aqueous acetic acid. Fractions were collected in 10 ml aliquots and the column effluent monitored at 280nm. Fractions showing positive absorbance at 280nm were diluted with 0.1% trifluoracetic acid (TFA) in water and analyzed by high performance liquid chromatography (HPLC)(4). The major peak of absorbance at 214 nm was determined to have a retention time of 12.42 min. The Fractogel fractions that contained greater than 70% of this peak were pooled and labelled Fr:1. The Fractogel fractions that contained less than 70% but greater than 50% of this peak were

pooled and labelled FR:2.

Analytical HPLC (4) on Fr:1 showed the 12.42 min peak to constitute 87% of the total area. Fr:1 was further characterized by amino acid analysis (5), sequence determination (6), and determination of % peptide content (7). Peptide sequence analysis (6) was also performed on the peptide-resin to confirm the expected sequence of the peptide.

(1) Stewart & Young, Solid Phase Peptide Synthesis, 2nd edition (1984), Pierce Chemical Co.

(2) Merrifield, R.B. (1963), J. Amer. Chem. Soc. 85, pp. 2149-2154

(3) All Boc-amino acids obtained from Bachem Inc., Torrance, Calif.

(4) Analytical HPLC Conditions:
Buffer A: 0.1% TFA/Distilled Deionized Water
Buffer B: 0.1% TFA/HPLC grade Acetonitrile
Gradient Conditions: 10% 'B' to 50% 'B' over 20 minutes
Wavelength: 214nm
Flow: 1.0 ml/min
Column: Vydac 214TP54 C-4 Protein column, 250 x 4.5 mm

(5) Amino Acid analysis performed on a LKB 4150 ALpha Amino Acid Analyzer.

(6) Peptide sequence determination performed on an Applied Biosystems 470A Protein Sequencer.

(7) Peptide content determined from recovery on amino acid analysis of known amount of peptide.

(8) Boc is a chemical abbreviation for the tert-Butyloxcarbonyl alpha-amino protecting group. The functional group is removed by hydrolysis in 50% Triflouroacetic acid (TFA)/50% Dichloromethane (CH_2Cl_2) after the amino acid has been coupled to the growing peptide chain. This action exposes the amino terminus of the chain to allow the next amino acid to be effectively coupled.

In addition to the Boc protecting group on every amino acid, the side chains of some amino acids are further protected from attack by the chemistry of peptide synthesis. These protecting groups, listed in parenthesis on the accounting list, are all stable to the conditions of peptide synthesis, yet are easily removed from the amino acid during cleavage in hydrofluoric acid. Anisole (methylphenyl ether) acts as a nucleophilic scavenger during the HF cleavage step to prevent alkylation of the peptide by the liberated protecting group carbonium ions. The protecting groups for the amino acids listed are defined below:

O-Benzyl: Benzyl-ester; attached to the hydroxyl side chain of both serine and threonine to prevent the acylation or branching of the peptide chain.

MeObzl: 4-Methoxybenzyl; attached to the sulfhydryl group of cysteine to prevent its oxidation during peptide synthesis.

Cl-Z: 2-chlorobenzyloxycarbonyl; attached to the alpha-amino group of lysine to prevent the formation of side chain growth from this site on the peptide.

C. Polymerization of the Peptide of formula (II):

The peptide contained in the Fr:1 pool of part B was lyophilized to remove acetic acid and solubilized at 200 µgm/ml in 0.1 M sodium bicarbonate buffer pH 9.0. Aliquots of this peptide diluted to 20 µgm/ml in sodium bicarbonate buffer were used to coat microtiter wells for ELISAs shown in Table IV. For tests shown below in Tables I-III peptide was solubilized in water at 10 mg/ml and diluted in phosphate buffer pH 7.3 to 5 µgm/ml for coating microtiter wells. The peptide in Fr:1 exists primarily in a single form that is believed to be unoxidized monomer. Because the peptide of formula (II) contains two cysteines, however, it polyermizes upon solubilization in neutrual or basic aqueous buffer. The peptide used in ELISAs described below is a mixture of very small amounts of linear monomer, and larger amounts of cyclic monomer (formed by intramolecular disulfide bonding) and even larger amounts of polymers (formed by intermolecular disulfide bonding) of various sizes. Without wishing to be bound thereby, Applicants believe that the polymer forms are important for the reactivities described herein. The cyclic monomer form, while retaining a portion of the antigenicity of the polymer form, is believed to be less efficient in binding to the microtiter wells and is less suited as the solid phase component of the ELISA. The presumed cyclic monomer is revealed as a sharp peak at about 12.7 min retention time in HPLC analysis while the polymer is characterized as a broad peak at approximately 15.9 min retention time. Oxidation conditions may be altered with respect to

temperature, pH, peptide concentration, and the like as known to those skilled in the art to alter the proportion of monomer, cyclic monomer and polymer remaining in the preparation, or the size of polymers formed. Small amounts of so called deletion peptides (lacking one or more amino acids) and their oxidation forms may also be found in the peptide preparations used in the ELISA. but these minor impurities do not affect the use of the peptide.

EXAMPLE II

Synthesis and Characterization of Peptides (III) through (XI):

Synthesis of these peptides was accomplished using substantially the same classical Merrifield technique as described earlier for peptide (II). For peptide (III), Boc-serine resin with substitution of 0.92 mMole/gram was used. For peptide (IV), Boc-isoleucine resin with substitution of 0.8 mMole/gram was used. Synthesis of the Boc-serine and Boc-isoleucine resins was accomplished by the Gisin method as described by Stewart & Young (1).

A. For peptide (III) the peptide sequence 'IWGCSGKLICTTAVPWNAS' was synthesized using the following Boc-L-amino acids in twelve meq excess:

Amino Acid	Solvent
Boc-Ala	CH_2Cl_2
Boc-Asn/Hobt	DMF
Boc-Trp	10% DMF/Ch_2Cl_2
Boc-Pro	CH_2Cl_2
Boc-Val	CH_2Cl_2
Boc-Ala	CH_2Cl_2
Boc-(O-Bzl)-Thr	CH_2Cl_2
Boc-(O-Bzl)-Thr	CH_2Cl_2
Boc-(MeOBzl)-Cys	CH_2Cl_2
Boc-Ile	CH_2Cl_2
Boc-Leu	10% DMF/CH_2Cl_2
Boc-(Cl-Z)-Lys	CH_2Cl_2
Boc-Gly	CH_2Cl_2
Boc-(O-Bzl)-Ser	CH_2Cl_2
Boc-(MeOBzl)-Cys	CH_2Cl_2
Boc-Gly	CH_2Cl_2
Boc-Trp	10% DMF/CH_2Cl_2
Boc-Ile	CH_2Cl_2

B. For peptide (IV) the peptide sequence 'AVERYLKDQQLLGIWGCSGKLI' was synthesized using the following Boc-amino acids in 12 meq excess:

Amino Acid	Solvent
Boc-Leu	10% DMF/CH_2Cl_2
Boc-(Cl-Z)-Lys	CH_2Cl_2
Boc-Gly	CH_2Cl_2
Boc-(O-Bzl)-Ser	CH_2Cl_2
Boc-(MeOBzl)-Cys	CH_2Cl_2
Boc-Gly	CH_2Cl_2
Boc-Trp	10% DMF/CH_2Cl_2
Boc-Ile	CH_2Cl_2
Boc-Gly	CH_2Cl_2
Boc-Leu	10% DMF/CH_2Cl_2
Boc-Leu	10% DMF/CH_2Cl_2
Boc-Gln/Hobt	DMF
Boc-Gln/Hobt	DMF
Boc-(Bzl)-Asp	CH_2Cl_2
Boc-(Cl-Z)-Lys	CH_2Cl_2
Boc-Leu	10% DMF/CH_2Cl_2
Boc-(Br-Z)-Tyr	CH_2Cl_2
Boc-(Tosyl)-Arg	10% DMF/CH_2Cl_2
Boc-(Bzl)-Glu	CH_2Cl_2
Boc-Val	CH_2Cl_2
Boc-Ala	CH_2Cl_2

As with peptide (II), in addition to the Boc protecting group on every amino acid the side chains of some amino acids are further protected from attack by the chemistry of peptide synthesis. In addition to those protecting groups described for the amino acids in the peptide (II) synthesis, the following protecting groups for the amino acids unique to peptides (III) and (IV) were used:

Hobt: 1-hydroxybenzotriazole; used in equimolar amounts to glutamine and asparagine during coupling to prevent dehydration to the nitrile forms.

Tosyl: p-toluene sulfonyl; used to acylate the guanidine group in the side chain of arginine.

Bzl: beta-benzyl ester; blocks the carboxyl groups in the side chain of aspartic acid and glutamic acid.

BrZ: 2-bromobenzyloxycarbonyl; blocks the hydroxyl group in the side chain of tyrosine.

Peptides were cleaved from the resin, filtered, extracted with acetic acid and run through a Fractogel desalting column as in Example I. For peptide (IV), Fractogel fractions were analyzed by analytical HPLC and fractions containing at least 30% of the total absorption at 214 nm as the major peak migrating at approximately 14 minutes retention time were pooled. The pooled fractions were chromatographed on carboxymethyl cellulose equilibrated with 0.01 M ammonium acetate, pH 4.4. The column was eluted with a step gradient of ammonium acetate and the fraction eluting at 0.2M ammonium acetate was collected, lyophilized, and analyzed by analytical HPLC. The major peak migrating at 14 minutes retention time comprised between 30% and 40% of the total absorption at 214 nm and the material had an acceptable amino acid content. This material was resolubilized and used in ELISA as described for peptide (II).

For peptide (III), Fractogel fractions were likewise analyzed by analytical HPLC. Fractions containing at least 70% of the total absorption at 214 nm as the major peak migrating at approximately 12.99 minutes retention time were pooled, lyophilized, and analyzed by HPLC and for amino acid content. This material was resolubilized and used in ELISA as described for peptide (II).

When used in combination as the solid phase component in an ELISA, 1 microgram each of peptides (III) and 0.5 micrograms of peptide (IV) were used per microtiter well. The peptide was either dried onto the well at 37^{o}C or

"wet packed" onto the plate by incubation overnight at 4°C.

C. For peptide (V), Boc-serine resin was used as described for synthesis of peptide (III). Synthesis of (V) proceeded as described for synthesis of (III) through the addition of the C-terminal isoleucine of peptide (III). From that point on, for completion of the (V) sequence, the procedure for addition of the amino acids in the sequence AVERYLKDQQLLG in peptide (IV) was followed.

Peptide (V) was cleaved from the resin, filtered, extracted with acetic acid and run through a Fractogel desalting column as described in Example I. Fractogel fractions containing the major peak of absorption at 280 nm were pooled and labelled Fr:1. Fr:1 was analyzed for amino acid content and found to be acceptable. This fraction was lyophilized and used in ELISA as described for peptide (II).

D. Following similar procedures the peptides of formulas (I), (VI) through (XVI) were prepared.

E. Polymerization of Peptides (III), (IV), and (V):

Peptides (I) through (III), (V), and (VII) through (X) contain two cysteines. Accordingly, these peptides can polymerize and cyclize through oxidative disulfide bonding. The addition of the four amino acis at the C-terminal end of (III) apparently allows the cyclic form of the peptide to bind to the plastic in the ELISA assay. As a result, the cyclic form of (III) is more effective in solid phase ELISA than is (II) cyclic. The forms of the (II), (III) and (V) peptides used thus far in ELISA to test for HTLV-III antibody recognition have been typically

a mixture of linear monomer, cyclic monomer, dimer and polymer.

Peptides (IV), (VI) and (XI) contain only one cysteine. These peptides can form a dimer structure through disulfide bonding.

Under conditions of solubilization of peptides in preparation for ELISA (e.g., 0.1M sodium bicarbonate buffer pH 9.0) most of the sulfhydryl groups of peptides (II), (III), (IV), and (V) have been converted to the disulfide form.

EXAMPLE III

Preparation of Comparative Peptides

Following similar procedures to that of Examples II and III, for comparative purposes peptides having the following formulae were synthesized:

QLQARILAVERY (C-I),
AVERYLKDQQLLG (C-II),
LKDQQLLGIWGCS (C-III),
IWGCSGKLI (C-IV), and
LICTTAVPWNASWSN (C-VIII).

These peptides each have sequences corresponding to the sequence of the HTLV-III envelope but fail to conform to the teachings of this invention. Specifically, peptides having the formula (C-I) and (C-II) are sequences upstream from the amino end of the sequence of formula (I) i.e., CSGKLIC. Peptides having the formula (C-III) contain only the amino terminal portion of the sequence of formula (I). Peptides having the formula (C-IV) contain

the full sequence of formula (I), but for the carboxy terminal L.-cysteine residue. Peptides having the formula C-VIII are sequences downstream from the carboxy end of the sequence of formula (I), as will be described herein, each of these peptides fail to exhibit the desirable immunoreactive properties.

EXAMPLE IV

Preparation of ELISA Assay Kit and Procedure for Use:

Procedure #1 for ELISA: (Table IV)

(1) Coat ELISA plate with peptide- 1µg/50µl/well in 0.1M $NaHCO_3$ pH9.

(2) Let plates dry overnight uncovered at 37°C; then wash with PBS.

(3) Block plates with 300µl/well of 5%NCS-PBS for 2 hrs. at 37°C.

(4) Shake out blocking buffer and drain well.

(5) Add 50µl/well test antisera for 30 to 120 minutes at 37°C. (If the antisera is to be diluted, use T-wash.) We have used 1:2 to 1:100 dilutions.

(6) Shake out test antisera and wash plate six times with PBS-Tween20.

(7) Add 100µl/well 2nd antibody diluted 1:4000 with T-wash. 30 to 120 minutes at 37°C.

(8) Shake out 2nd antibody and wash plate six times with PBS-Tween20.

(9) Add 100µl/well OPD substrate (or 5 µl ABTS solution) for 20 minutes at RT.

(10) Add 50µl/well of 4N H_2SO_4 to stop OPD reaction (or 100 µl/well of 1% SDS for ABTS reaction).

(11) Read plate on an MR 600 Microplate ELISA plate reader. (490nm for OPD or 405 nm for ABTS)

Reagents:

(1) 0.1M NaHCO3 pH9.

(2) PBS + 5% Normal Calf Serum.

(3) T-wash : (780ml TBS + 20ml NCS + 1.6gm BSA + 0.4ml Tween20)

TBS 12.11g Tris Base
17.5g NaCl
1800ml H_2O
pH to 7.6 with HCl (Ca.3N)
final volume at 2000ml

(4) Washing buffer / PBS-Tween20: 0.5ml Tween10/1L PBS.

(5) OPD substrate: 1OPD tablet/3ml H_2O/1.24ul 30%H_2O_2.

(6) 4N H_2SO_4.

(7) ABTS: H_2O_2 in 1:1 ratio by volume of solutions supplied by Kirkegaard and Perry Laboratories, Inc., Gaithersbury, Md.)
ABTS = 2,2'-azino-di-[3-ethyl-benzthiazoline sulfonate]

(8) 1% SDS.

The material used in step 1 for coating the ELISA plate is peptide of formula (II), (III), (IV), (V), or a mixture thereof as described above. The second antibody is either a commercially-available peroxidase-labelled, polyclonal antibody (Cappel Laboratories Catalog No. 3201-0231; Peroxidise-conjugated IgG fraction of goat anti-human immunoglobulins) or a peroxidase-labelled mouse monoclonal anti-human IgG antibody, or a mixture of peroxidase-labelled mouse monoclonal anti-human IgG, IgA and IgM antibodies.

Procedure #2 for ELISA (Tables I-III):

(1). Coat ELISA plate with peptides- µgm peptide (IV), 0.5 µgm peptide (III) 200µl/well in 0.1M carbonate buffer, pH 9.6.

(2). Incubate plates overnight at 4°C.

(3). Block plates with 300 µl/well 1.0% BSA-PBS plus additives for 2 hr at 37°C.

(4). Shake out blocking buffer.

(5). Dry plates for 1.5 hr at 37°C.

(6). Add 200 µl/well 1% Bovine Gamma Globulin - 5% BSA - 0.5% Tween-PBS, pH 7.2.

(7). Add 10 µl/well test sera, incubate 30 min at 37°C.

(8). Shake out test sera, wash plate 5x with PBS-0.5% Tween.

(9). Add 200 µl/well monoclonal anti-human IgG diluted 1:3500 with 50% fetal calf serum-1% horse serum-0.5% Tween-PBS.

(10). Incubate 30 min at 37°C.

(11). Shake out test sera, wash plate 5x with PBS-0.05% Tween.

(12.) Add 200 µl/well OPD substrate and incubate for 30 min at room temperature.

(13). Add 50 µl/well 4 N H_2SO_4 to stop reaction.

(14.) Read plate at 490 nm in MR 600 Microplate Reader.

Reagents:

(1). Phosphate Buffered Saline (PBS) pH 7.3
8.0 gm of sodium chloride
0.2 gm of potassium phosphate, monobasic
1.16 gm of sodium phosphate, dibasic
0.2 gm of potassium chloride
0.2 gm of thimerosal
water to 800 ml and mix
adjust pH if necessary, add water to 1 L

(2). Coating Buffer, pH 9.6
0.01M carbonate buffer.

(3). Blocking buffer (1% BSA-PBS plus additives)
1% Bovine Serum Albumin (Signa #A7030)
10 Kµ/ml Aprotinin
10µg/ml trypsin inhibitor
10 nM EACA (E-amino caproic acid)
0.5 mM PMSF (phenyl-methyl-sulfonyl flouride)
2.0 mM EDTA
10% glycerol

(4). Specimen Diluent

10.0 gm Bovine Gamma Globulin, Fraction II, lyophilized
50.0 gm Bovine Albumin, Fraction V
0.5 ml Polysorbate 20 (Tween 20)
Add water to 1 L and mix
Filter through 0.2 micron filter.

(5). Conjugate Diluent

490 ml PBS
500 ml heat inactivated fetal bovine serum
10 ml heat inactiviated horse serum
0.1 g thimerosal
0.329 g potassium ferricyanide
water to 1 L. mix, filter through 0.2 micron filter

EXAMPLE V

The ELISA kits described in Example IV-procedure 1 made with peptide (II) were evaluated against a panel of sera comprising sera from normal subjects, patients with disorders or diseases unrelated to AIDS, known AIDS patients, known ARC patients, and patients whose diagnosis is unknown but who are antibody positive by commercial tests or by Western blot assay. The results are summarized in Tables I - IV. For comparison, these same sera samples were assayed with commercially available kits and by Western blot assay. The commercial kits selected for these studies were from Abbott Laboratories, North Chicago, Ill. and Electro-Neucleonics, Inc. (ENI), Columbia, Md.

A. Table 1 shows results with normal sera.

TABLE I

Assay of Normal Sera by ELISA using peptide (II).

Number of Samples	E8 Assay	ENI Assay	Abbott Assay	Diagnosis/ Sample I.D.
198	-	5-,194NT	41-,156NT	Normal
1	-	-	+	Normal/749
1	+	NT	NT	Normal/2846
200				

Mean of Normals = 0.016 for E8 assay
Standard Deviation (S.D.) from themean = 0.017
Cutoff Value at Mean + S.D. = 0.104
False Positive rate in 200 samples at 0.104 cutoff = 0.5% (1/200)
NT = Not Tested

As is shown in the above table, subject sera not containing antibodies to HTLV III generally do not react to peptide (II) in a standard ELISA. This allows for calculation of an absorption cutoff value to distinguish between antibody negative and antibody positive sera. In the above assay of 200 normal sera, a cutoff value of 0.104 was selected. At this cutoff the false positive rate is expected to be less than or equal to 0.5%.

B. To indicate the superior effectiveness for eliminating false positives by employing the peptide of formula (II) over the commercially available kits using viral lysate, a number of sera from patients with two disorders unrelated to AIDS, namely Naso-Pharyngeal Carcinoma and Rheumatoid Arthritis, were tested by the subject assay and commercial tests. The results are summarized in Table II below and indicate the increased specificity of the subject assay. Many samples which gave

false positive results with commercial tests correctly identified as negative by the subject assay.

TABLE II

Test of Naso-Pharyngeal Carcinoma and Rheumatoid Arthritis patients.

Number of Samples	Subject assay	ENI assay	Abbott assay	Diagnosis: Sample I.D.
6	(+)	NT	(+)	NPC/NON-AIDS
17	-	NT	(+)	NPC/NON-AIDS
8	-	NT	-	NPC/NON-AIDS
1	-	NT	NT	NPC/NON-AIDS;920
1	-	(+)	-	RA/NON-AIDS;305
7	-	2-,5NT	3-,4NT	RA/NON-AIDS
1	-	-	(+)	RA/NON-AIDS;615

(+) = False Positives NT = NOT TESTED

NPC = Naso-Pharyngeal Carcinoma; RA = Rheumatoid Arthritis

C. To indicate the effectiveness of the subject assay for detection of HTLV-III antibody in AIDS/ARC patient sera compared to commercial kits, the ELISA kit described in Example IV-Procedure 1 was evaluated against a panel of sera derived from diagnosed AIDS and ARC patients. The results are summarized in Table III and show that the assay is equivalent to commercial kits for its ability to identify sera containing antibody to HTLV-III.

TABLE III

Assay of AIDS/ ARC sera:

Number of Samples	Subject assay	ENI assay	Abbott assay	Diagnosis/ Sample I.D.
67	+	+	+ (16NT)	AIDS
2	(-)	(-)	(-)	AIDS/533,3621
1	(-)	+	+	AIDS/653
2	+	+	(-)	AIDS/661,662
21	+	+	+	ARC
2	(-)	(-)	(-)	ARC/512,529
95				

(-) = False Negatives
ARC = Aids Related Complex

D. The high rate of false positives characteristic of presently available kits using viral lysate is due in part to the presence of cellular antigens in the lysate that react with antibodies present in both AIDS and non-AIDS patient sera. Additionally, complex antigens such as those derived from a virus such as HTLV-III contain many epitopes and are more likely to react non-specifically with antibodies present in human sera. The subject peptide (II) reduces the complexity of the antigen used to react with patient sera down to one or perhaps only a few epitopes. The chance of non-specific interaction with non-HTLV-III antibodies is expected to be greatly reduced. Non-specific interactions, however, may still occur since some antibodies are "sticky" and can bind to the plastic support used in the assay, or to other proteins such as bovine serum albumin or goat sera used to block the plate after addition of the peptide. Presented

in Table IV below are data relating to the assay of various patient sera. In addition to the usual assay with peptide (II) as described in Example IV-Procedure 1 we have assayed each sera against peptide (II) after mixing said sera samples with an effective blocking amount of the peptide (II). Also included in the Table are the results of assaying each sample with commercially available kits.

TABLE IV

Competition Assay Confirming Positive/Negative ELISA:

Samp. I.D.	ELISA Value & Score with Peptide Not Blocked	Blocked (Score)	Abbott Assay	ENI Assay	Western Assay	Diag.
615	0.031	0.033(-)	-	-	-	RA
3195	0.026	0.045(-)	-	+*	-	DP
3196	0.028	0.039(-)	-	+*	-	DP
3197	0.065	0.073(-)	-	+*	-	DP
3376	0.104	0.105(-)	-	+*	-	UNK
3362	0.740	0.727(-)	-	+*	-	UNK
912	0.055	0.065(-)	+*	NT	-	NPC
918	0.100	0.092(-)	+*	NT	-	NPC
922	0.127	0.103(-)	+*	NT	NT	NPC
923	0.114	0.080(-)	+*	NT	NT	NPC
3644	0.336	0.380(-)	+*	+*	-	UNK
3406	1.400	1.390(-)	+*	+*	-	DP
3461	1.426	1.137(-)	+*	+*	-	DP
3532	1.770	0.080(+)	+	+	+	UNK
3469	0.300	0.030(+)	+	+	+	UNK
3431	0.507	0.087(+)	NT	+	+	UNK
644	0.160	0.024(+)	NT	+	+	AIDS
659	0.510	0.042(+)	+	+	+	AIDS
661	0.500	0.031(+)	-**	+	+	AIDS
662	0.160	0.030(+)	-**	+	+	AIDS

* = False Positive
** = False Negative
UNK = Unknown
RA = Rheumatoid Arthritis, Non-Aids
DP = Dialysis Patient, Non-Aids
NPC = Naso-Pharyngeal Carcinoma, Non-Aids

It is evident from these results that non-AIDS sera samples incorrectly identified as positive by either or both of the commercially available kits are correctly identified as negative using the peptide competition assay. Furthermore, even samples incorrectly identified as positive by the assay are correctly identified as negative by the peptide competition assay. Significantly, the blocking or competition assay also serves as a confirmatory assay in tests of sera samples that do contain antibodies to HTLV-III. The last seven samples tested as shown in Table IV are positive for antibody by both the subject test and commercially available assays (with the exception of two false negatives using the Abbott kit) and by Western blot assay. The reactivity of these sera with peptide (II) is effectively blocked by mixing the sera with peptide (II) indicating that the reactivity of antibody to peptide is peptide specific and that these last seven samples are true positives.

EXAMPLE VI

ELISA kits as described in Example IV-Procedure 1 were made with peptide formulas (I) through (XV) as well as formulas (C-I) through (C-VIII). The ELISA kits were each evaluated against a ten sample panel of sera comprising clinically positive samples, i.e., samples which were symptomatic of the HTLV-III infection as determined by commercial assays and western blot assay. The results of these tests are shown in Table V below.

A peptide assay is considered positive if the absorbent level of the ELISA test was more than twice the background level as determined by averaging the absorbence of two normal sera. The mean reported represents the mean optical density level of the ELISA as calculated after the

background level was subtracted. The index reported is a weighted activity of a given peptide relative to the activity of the peptide of formula (II). The index is weighted in favor of the ability of a particular peptide assay to correctly report a positive value as distinguished from the level of the background normal response. The formula for deriving the index is as follows:

$$-\frac{(\%\ \text{Positive})^3 \times \sqrt{\text{MEAN}}}{(\%\ \text{Positive})_{II})^3 \times \sqrt{\text{MEAN}_{II}}} = \text{Index}$$

wherein: % Positive is % Positive for the given peptide assay;

MEAN is MEAN for the given peptide assay;

% Positive_{II} is % Positive for peptide assay having the Formula (II); and

% MEAN_{II} is MEAN for the peptide assay having the Formula (II).

TABLE V

PEPTIDE FORMULA	SEQUENCE	%POSITIVE	MEAN	INDEX
(II)	IWGCSGKLICTTAVP	80	1.67+0.82	1.00
(C-V)	QLTVWGIKQLQARIL	0	- -	0
(C-VI)	GIKQLQARILAVERY	20	0.45+0.06	0.01
(C-I)	QLQARILAVERY	0	- -	0
(C-IX)	QARILAVERYLKDQQ	0	- -	0
(XV)	RILAVERYLKDQQLLGIWGCS	90	2.01+1.40	1.56
(C-II)	AVERYLKDQQLLG	10	0.04 -	<0.01
(C-VII)	AVERYLKDQQLLGIW	60	1.18+0.68	0.35
(IV)	AVERYLKDQQLLGIWGCSGKLI	100	2.23+0.68	2.26
(XII)	AVERYLKDQQLLGIWGCSGKLIC	100	1.95+0.27	2.11
(C-III)	LKDQQLLGIWGCS	30	0.28+0.06	0.02
(XIV)	LKDQQLLGIWGCSGK	90	1.69+0.65	1.43
(VI)	LKDQQLLGIWGCSGKLI	100	1.19+0.85	1.65
(VII)	LLGIWGCSGKLIC	50	0.22+0.05	0.09
(XI)	LLGIWGCSGKLICTT	80	1.53+0.77	0.96
(C-IV)	IWGCSGKLI	20	0.17+0.00	0.01
(I)	CSGKLIC	60	0.34+0.11	0.19
(VIII)	QQLLGIWGCSGKLICTTAVPWNAS	90	0.51+0.25	0.79
(IX)	IWGCSGKLICTTAVPWN	70	0.86+0.45	0.48
(III)	IWGCSGKLICTTAVPWNAS	100	2.20+0.86	2.24
(XIII)	GCSGKLICTTAVPWN	100	2.14+0.58	2.21
(X)	CSGKLICTTAVPWNAS	100	1.74+1.01	1.99
(XI)	SGKLICTTAVPWNAS	50	0.86+0.63	0.17
(C-VIII)	LICTTAVPWNASWSN	0	- -	0
(V)	AVERYLKDQQLLGIWGCSGKLICTTAVPWNAS	100	1.53+0.66	1.89
(III) & (IV)	AVERYLKDQQLLGIWGCSGKLI + IWGCSGKLICTTAVPWNAS	100	>3.0 -	2.62

As can be seen from the above Table, assays made from peptides having the Formulas (I) through (XV), all provide positive results of at least 50% or greater and in each case manifest an index of at least 0.1. On the other hand, each of the comparative segments, although representing closely adjacent or overlapping or partially overlapping segments from the HTLV-III envelope, fail to exhibit such positive results or such high index.

EXAMPLE VII

Additional AIDS/ARC patient sera were tested with ELISA assays employing the two highly reactive peptides of formulas (III) and (IV) (Example IV-Procedure 1). The reactivity, expressed as absorbence values, of some of these sera is shown in Table VI.

TABLE VI

	SERUM SAMPLE	Peptide Formula (III)	Peptide Formula (IV)
	3362	0.503	0.151
	3412	>2.00	0.540
A.	3693	0.559	0.120
	3722	0.620	0.193
	0649	1.756	0.379
	3544	0.428	>2.00
	3575	0.350	1.773
	3744	0.311	1.326
B.	3790	0.224	1.765
	0509	0.403	2.000
	0653	0.111	0.999
	0662	0.301	1.392
	3416	1.914	>2.00
	3456	1.670	1.780
C.	3666	>2.00	>2.00
	3414	1.453	1.057
	3411	>2.00	>2.00
	3413	>2.00	>2.00

Most sera which were tested reacted very well with both peptides, much as is seen with samples in Table VI, group C. Occassionally, however, some samples were far more reactive with one peptide than the other, as shown in Table VI, groups A and B. These data indicate that there may be more than one epitope (e.g. a linear and a conformational epitope) in this thirty-two amino acid region that is commonly recognized by patients that have

been exposed to AID virus. High performance liquid chromatography (HPLC) analysis of peptides of formulas (III) and (IV) in solution indicate that formula (III) peptides exist largely as cyclic monomers, while formula (IV) peptides are mostly in dimer form. The structural characteristics imparted to these two peptides by disulfide bonding may relate to both antigen presentation and the creation of a conformational epitope.

EXAMPLE VIII

Further evidence that more than one epitope is present in formula (III) and (IV) peptides can be deduced from competition studies, the results of an example of which are shown in Table VIIA. In this study, formula (III) and (IV) peptides were both applied to a microtiter plate as immobilized antigen and an ELISA assay was performed under five different conditions: without competition, or competition with excess formula (III) peptide, formula (IV), both peptides, or an heterologous peptide. The competition was performed by adding the appropriate peptide(s) to the diluted serum just before adding the serum sample to the microtiter well.

TABLE VIIA

		COMPETING PEPTIDE FORMULA			
SAMPLE	UNBLOCKED	(III)	(IV)	(III)/(IV)	HETEROLOGOUS
3412	1.450	0.113	1.182	0.059	1.348
3413	>2.00	0.381	2.032	0.068	2.026
3416	>2.00	1.811	1.055	0.197	2.041
3544	1.810	0.876	0.137	0.048	1.750
3575	1.267	0.982	0.105	0.036	1.304
3693	0.340	0.103	0.217	0.065	0.293
3790	1.558	1.320	0.635	0.134	1.349

Under these conditions it is very clear that some samples react very well with each or both of the subject peptides. For example, sample 3416 reacts well with both peptides, since competition with the formula (III) peptide gives an optical density (OD) of >1.8, competition with the formula IV peptide yields an OD of 1.055 and, in the presence of both competing peptides, the OD goes down essentially to background levels. Other serum samples such as 3412 react much more strongly with one peptide than the other; in this case, the OD is reduced from >1.4 down to 0.113 when blocked with formula (III) peptide and remains >1 when competed with formula (IV) peptide. However, there is clearly some reactivity with formula (III) peptide because, in the presence of both peptides, immunoreactivity is completely abolished (0.059).

Another competition study with the formula (I) peptide on the plate, shows that formula (IV) peptide does not effectively compete with formula (I) peptide although formula (III) peptide competes very effectively. See Table VIIB below.

TABLE VIIB

		COMPETING PEPTIDE FORMULA			
SAMPLE	UNBLOCKED	(I)	(IV)	(III)/(IV)	HETEROLOGOUS
013	1.177	0.038	0.046	0.770	0.865
014	0.383	0.050	0.067	0.273	0.324

Thus, it appears from these analyses that an epitope present in formula (III) peptide is also present on formula (I) peptide, and that this epitope is substantially different from those present in formula (IV) peptide. Furthermore, based on this same sample, virtually all sera react with the epitopes presented on the formula (III) and (IV) peptides, although in many cases, more strongly with one peptide than with the other.

EXAMPLE IX

Using 1µg of combination of formula (III) and 0.5 µg of formula (IV) peptides as the solid phase antigen in an ELISA assay (Example IV-Procedure 2), the specificity and sensitivity of this assay was equal or superior to any of the commercially available viral lysate antibody detection kits tested.

Table VIII presents ELISA results obtained using patient sera, normal sera, and sera from miscellaneous disease groups that include rheumatoid arthritis, naso-pharyngeal carcinoma (NPC), Epstein-Barr virus infection, cytomegalovirus infection, gram negative sepsis, toxoplasma gondii, systemic lupus erythematosus, and herpes virus infections.

TABLE VIII

SAMPLE GROUP	NUMBER OF SERA	ENI$^+$	(III)/(IV)$^+$	ENI$^-$	(III)/(IV)$^-$
AIDS + ARC	458(243)*	449	450	9	8
SYPTOMATIC PLS	320(146)	239	242	81	78
ASYMPTOMATIC/HIGH RISK IMMUNE ABNORMALITIES	135(87)	38	39	97	96
ASYMPTOMATIC/HIGH RISK IMMUNE NORMAL	134(69)	10	10	124	124
NORMAL/NON-AIDS	728(728)	12	4	716	724
MISCELLANEOUS DISEASE GROUPS/NON-AIDS	387(387)	10	7	377	380
TOTAL NON-AIDS	1115	22	11	1093	1104

*Indicates number of patients.

When bona fide normal sera were tested for reactivity in the (III)/(IV) assay and in the assay marketed by Electronucleonics, Incorporated (ENI), the (III)/(IV) peptide assay has a significantly lower false positive rate. As Table VIII shows, the false positive rate for ENI was 1.65% (12/728), while the (III)/(IV) peptide assay has a false positive rate of only 0.55% (4/728). When the false positive rate in the Miscellaneous Disease Group is examined, the peptide assay had a slightly lower false positive rate than did the ENI assay, 1.81% vs. 2.58% (7/387 vs. 10/387).

The above examples have been given only for illustration purposes and not to limit the scope of the

present invention which scope is defined only in the appended claims.

WHAT IS CLAIMED IS:

1. A synthetic peptide including a sequence selected from the group consisting of:

CSGKLIC,
IWGCSGKLICTTAVP,
IWGCSGKLICTTAVPWNAS,
AVERYLKDQQLLGIWGCSGKLI,
AVERYLKDQQLLGIWGCSGKLICTTAVPWNAS,
LKDQQLLGIWGCSGKLI,
LLGIWGCSGKLIC,
QQLLGIWGCSGKLICTTAVPWNAS,
IWGCSGKLICTTAVPWN,
CSGKLICTTAVPWNAS,
SGKLICTTAVPWNAS,
AVERYLKDQQLLGIWGCSGKLIC,
GCSGKLICTTAVPWN,
LKDQQLLGIWGCSGK,
RILAVERYLKDQQLLGIWGCS,

the antigenic and immunologic fragments and pharmaceutically or diagnostically acceptable salts thereof.

2. The synthetic peptide of claim 1 having the formula:

CSGKLIC

3. The synthetic peptide of claim 1 having the formula:

IWGCSGKLICTTAVP

4. The synthetic peptide of claim 1 having the formula:

IWGCSGKLICTTAVPWNAS

5. The synthetic peptide of claim 1 having the formula:

AVERYLKDQQLLGIWGCSGKLI

6. The synthetic peptide of claim 1 having the formula:

AVERYLKDQQLLGIWGCSGKLICTTAVPWNAS

7. The synthetic peptide of claim 1 having the formula:

LKDQQLLGIWGCSGKLI

8. The synthetic peptide of claim 1 having the formula:

LLGIWGCSGKLIC

9. The synthetic peptide of claim 1 having the formula:

QQLLGIWGCSGKLICTTAVPWNAS

10. The synthetic peptide of claim 1 having the formula:

IWGCSGKLICTTAVPWN

11. The synthetic peptide of claim 1 having the formula:

CSGKLICTTAVPWNAS

12. The synthetic peptide of claim 1 having the formula:

SGKLICTTAVPWNAS

13. The synthetic peptide of claim 1 having the formula:

AVERYLKDQQLLGIWGCSGKLIC

14. The synthetic peptide of claim 1 having the formula:

GCSGKLICTTAVPWN

15. The synthetic peptide of claim 1 having the formula:

LKDQQLLGIWGCSGK

16. The synthetic peptide of claim 1 having the formula:

RILAVERYLKDQQLLGIWGCS

17. The peptide of claim 1 in polymeric form.

18. In combination, a mixture of two or more peptides having enhanced recognition for antibodies to HTLV-III virus as compared to each peptide taken alone, said mixture comprising selecting two or more peptides containing amino acid residue sequences homologous to portions of the HTLV-III virus gp 41 protein sequence wherein:

a first of said peptides comprises the portion of the HTLV-III virus gp 41 protein sequence CSGKLIC; and

a second of said peptides comprises the sequence ZLLGXWZ'; wherein X is selected from the group consisting of I, L, M or F; Z is selected from the amino acid residue sequence of the HTLV-III virus gp 41 protein immediately adjacent to the amino side of the L-leucine residue in the 599th position of the gp 41 protein; Z' is selected from the amino acid residue sequence of the HTLV-III virus gp 41 protein immediately adjacent to the carboxy side of the L-tryptophan residue in the 603rd position of the gp 41 protein; wherein one of Z and Z' may be zero residues

long; and wherein Z and Z' together comprise at least ten residues.

19. The mixture of peptides of claim 18 wherein:

said first peptide is selected from the group consisting of:

IWGCSGKLICTTAVPWNAS;
GCSGKLICTTAVPWN;
CSGKLICTTAVPWNAS; and

said second peptide is selected from the group consiting of

AVERYLKDQQLLGXWGCSGKLI, and
AVERYLKDQQLLGXWGCSGKLIC.

20. The mixture of peptides of claim 19 wherein said first peptide consists of

IWGCSGKLICTTAVPWNAS, and

said second peptide consists of

AVERYLKDQQLLGXWGCSGKLI.

21. The mixture of claim 20 wherein X is I.

22. The mixture of peptides of claim 19 wherein said first peptide consists of

GCSGKLICTTAVPWN, and

said second peptide consists of

AVERYLKDQQLLGXWGCSGKLI.

23. The mixture of claim 22 wherein X is I.

24. A method for detecting or determining antibodies to HTLV-III in a fluid suspected of containing said antibody which comprises:

a) providing at least one peptide of claim 1 attached to a solid surface;

b) contacting said peptide coated solid surface with the fluid to be tested for a sufficient time to allow an immunologic reaction to occur; and

c) detecting or determining the presence or amount of antibodies bound to said peptide or antigenic fragment on the surface of said solid.

25. The method of claim 24 wherein the detecting or determining step comprises contacting said surface with labelled anti-human immunoglobulin.

26. A method of preparing antibodies to HTLV-III virus which comprises immunizing an animal with an immunogenically-effective amount of at least one peptide of claim 1, polymerized and/or attached to a suitable immunogenic carrier and bleeding said animal.

27. A method of immunizing an animal against HTLV-III virus which comprises parenterally administering to said animal an immunogenically-effective amount of at least one

peptide of claim 1 polymerized and/or attached to an immunogenically-effective carrier.

28. A method of treating an animal having latent or actual HTLV-III virus infection which comprises parenterally administering to said animal an immunogenically-effective amount of at least one peptide of claim 1 polymerized and/or attached to an immunogenically-effective carrier.

29. A diagnostic kit for detecting or determining antibodies to HTLV-III which comprises: (a) a solid surface having bound thereto at least one peptide of claim 1; and (b) labelled anti-human immunoglobulins.

30. A therapeutic or prophylactic composition of matter comprising an immunogenically-effective amount of at least one peptide of claim 1 polymerized and/or attached to an immunogenically-effective carrier.

31. A sandwich method for detecting or determining HTLV-III virus or viral-specific antigen in a fluid suspected of containing said virus or antigen which comprises:

a) providing antibody to at least one peptide of claim 1 attached to a solid surface;

b) contacting said antibody-coated solid surface with the fluid to be tested for a sufficient time to allow an immunologic reaction to occur; and

c) detecting or determining the presence or amount of HTLV-III virus or viral specific antigen attached to said antibodies on said solid surface.

32. A competition method of detecting or determining HTLV-III virus or viral specific antigen in a fluid suspected of containing said virus or antigen comprising the steps of:

a) providing an antibody to at least one peptide of claim 1, said antibody being attached to a solid surface;

b) mixing an aliquot of the fluid to be tested with a known amount of labelled subject peptide to produce a mixed sample;

c) contacting said antibody - coated solid surface with said mixed sample for a sufficient time to allow an immunologic reaction to occur;

d) separating the solid surface from the mixed sample;

e) detecting or determining the presence or amount of labelled peptide either bound to the solid surface or remaining in the mixed sample; and

f) determining from the result in step e) the presence or amount of said virus or antigen in said fluid.

33. A diagnostic kit for detecting or determining HTLV-III virus or viral specific antigen which comprises:

a) a solid surface having bound thereto antibody to at least one peptide of claim 1; and

b) a known amount of labelled antibody to HTLV-III, to a viral specific antigen, or to a peptide of claim 1.

34. A method of determining the accuracy of a positive result in a test for HTLV-III antibodies on a fluid sample suspected of containing said antibodies, said test being a sandwich assay in which a recombinant HTLV-III protein or synthetic HTLV-III peptide or antigenic fragment thereof is attached to the solid surface, said method comprising the steps of:

a) mixing an aliquot of said sample with an effective blocking amount of the said recombinant HTLV-III protein or synthetic HTLV-III peptide or antigenic fragment thereof to produce a mixed sample;

b) contacting the mixed sample with the solid phase of said test for a sufficient time to allow an immunologic reaction to occur;

c) determining whether binding of said antibodies in said mixed sample with said solid phase is inhibited compared to said binding in the absence of said peptide, fragment, or protein; and

d) determining, based on the presence or absence of said inhibition, the accuracy of said positive result.

35. A diagnostic kit for determining or detecting antibody to HTLV-III and confirming the positive result which comprises:

a) a solid surface having bound thereto a peptide of claim 1;

b) an effective blocking amount of the said peptide of claim 1; and

c) labelled anti-human immunoglobulin.

36. A method for preparing monoclonal antibodies to HTLV-III comprising: a) immunizing a host animal with a peptide of claim 1 in polymerized form or attached to a suitable immunogenic carrier; b) isolating the splenocytes from said immunized host; c) fusing said splenocytes with a suitable myeloma cell line; d) selecting the fused cells by reactivity to the immunizing peptide, to HTLV-III, or to HTLV-III-infected cells; e) either culturing the selected hybridoma <u>in vitro</u> or injecting it into a suitable host; and f) recovering the desired monoclonal antibody from the supernatant over the cultured hybridoma or from the serum or ascites of the injected host.

INTERNATIONAL SEARCH REPORT

International Application No PCT/US87/00577

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(4) G01N 33/53,531,532,543,571,574,577,569

U.S. Cl. 424/86,89;435/5,240;436/518,548,808,813;530/324,326,327

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
U.S.	424/86,89;435/240;436/518,548,808,813;530/324,326,327

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
Y	US, A, 4,134,792 (BOGUSLASKI) 16 January 1979, see column 9, line 30, column 11, line 15.	31,33
Y,P	US, A, 4,629,783 (COSAND) 16 December 1986, see column 2, lines 9-13 and 50-56; column 3; column 5, lines 25-35; column 6, lines 6-14, and 46 to column 7, line 56; and column 9, lines 37-49.	1-36
Y	A. FETEANU, "Labelled Antibodies in Biology and Medicine", published 1978, by Abacus Press (England), pages 109-111, especially see page 111.	34-35
Y	L. HUDSON, et al., "Practical Immunology", published in 1980, by Blackwell Scientific Publications (London), pages 5-9, especially see page 9.	26

* Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
02 JUNE 1987	22 JUN 1987
International Searching Authority [1]	**Signature of Authorized Officer** [20]
ISA/US	Jack Spiegel

Form PCT/ISA/210 (second sheet) (May 1986)

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

Y	Diagnostic Horizons, Volume 2, no. 1, issued 1978 February (Walkersville, Maryland), A. Voller, "The Enzyme Linked Immunosorbent Assay (ELISA)", pages 1-7, especially see page 2.	34-35
Y	Nature, Volume 313, issued 1985 February (London), M.A. Muesing, et al., "Nucleic Acid Structure and Expression of The Human AIDS/Lymphadenopathy Retrovirus", pages 450-458, see pages 453 and 455, Figure 5.	1-36

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [10]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers .... because they relate to subject matter [12] not required to be searched by this Authority, namely:

2. ☐ Claim numbers..... , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [13], specifically:

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [11]

This International Searching Authority found multiple inventions in this international application as follows:

I. Claims 1-26, 29 and 31-36 drawn to synthetic peptides and diagnostic assays using same; Class 436/518.

II. Claims 27-28 and 30 drawn to method of immunizing and body treating with kit therefor; Class 424/89.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application. Telephone Practice.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No [18]
Y	New England Journal of Medicine, Volume 312 No. 5, issued 1985 January (Boston), J. Schupbach, et al., "Antibodies to HTLV-III in Swiss Patients with Aids and Pre-Aids and in Groups at Risk for Aids", pages 265-270, see pages 267 and 268.	1-36
Y	Nature, Volume 256, issued 1975 (London), G. Kohler, et al., "Continuous Cultures of Fused Cells Secreting Antibody of Pre-defined Specificity", pages 495-497, see pages 496-497.	36
Y,P	Proceeding National Academy of Sciences USA, Volume 83, issued 1986 August (Washington, D.C.), J.J. Wang, et al., "Detection of Antibodies to Human T-lymphotropic Virus Type III by using a Synthetic Peptide of 21 Amino Acid Residues Corresponding to a Highly Antigenic Segment of gp 41 Envelope Protein", pages 6159-6163, see entire document.	1-36

Attachment to Form PCT/ISA/210 Part VI. 1

Telephone approval:

$140 payment approved by Richard Grochala on 21 April 1987 for Group II; charge to Deposit Account No. 10-750. Counsel advised that he has no right to protest for any group not paid for and that any protest must be filed no later than 15 days from the date of mailing of the search report (Form 210).

Reasons for holding lack of unity of invention:

The invention as defined in Group I (claims 1-26, 29, and 31-36), drawn to synthetic peptides and assays using same which is classified in Class 436, Subclass 518, may be used in materially different processes than the invention of Group II (claims 27-28 and 30), drawn to methods of immunizing and body testing with kit therefor and classified in Class 424, Subclass 89, such as use in diagnostic assays.

Time Limit for Filing a Protest

Applicant is hereby given 15 days from the mailing date of this Search Report in which to file a protest of the holding of lack of unity of invention. In accordance with PCT Rule 40.2 applicant may protest the holding of lack of unity only with respect to the group(s) paid for.